

(19)



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European Patent Office
Office européen des brevets



(11)

EP 1 241 479 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:
18.09.2002 Bulletin 2002/38

(51) Int Cl.⁷: **G01N 33/74, G01N 33/543**

(21) Application number: **01106463.1**

(22) Date of filing: **23.03.2001**

(84) Designated Contracting States:
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR**
Designated Extension States:
AL LT LV MK RO SI

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(30) Priority: **12.03.2001 EP 01105418**

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(54) **Method of determining urotensin II in body fluids and diagnosis of cardiovascular diseases**

(57) Method of determining the biological active urotensin II in a sample of a body fluid, comprising contacting the sample with polyclonal and/or monoclonal antibodies which have been elicited by immunisation with a cyclic peptide having the sequence:

ETPDCFWKYC-βAla-C,

SEQ ID NO: 2

having a disulfide bridge formed between Cys⁵ and Cys¹⁰, and comprising the artificial sequence βAla-Cys, optionally conjugated with a carrier peptide. The obtained antibodies are then either purified or characterised by their binding to cyclic human urotensin II and can be used in a binding assay for a diagnosis of cardiovascular diseases, hepatorenal syndrome, cirrhosis, diseases involving haemodynamic alterations, pathological conditions involving vasoconstrictive substances.

Description

[0001] The invention relates to methods of determining urotensin II in body fluids and diagnosis of cardiovascular and vascular diseases.

[0002] Urotensin II (U II) is a somatostatin like-peptide initially isolated from the caudal neurosecretory system of teleost fish [Pearson D et al. (1980) PNAS U.S.A., 77(8), 5021-4]. The cDNA of the urotensin II precursor has been cloned from a number of species, including human, mouse, rat and frog [WO 99/35266 (PCT/US99/00489); US 6,075,137; Coulouarn Y et al. (1998), PNAS U.S.A., 95(26), 15803-8; Coulouarn Y et al. (1999), FEBS Lett., 457, 28-32; Ames RS et al. (1999), Nature 401, 282-6]. The human urotensin II precursor consists of 124 amino acid residues which is proteolytically cleaved to generate a bioactive peptide of 11 residues (Swiss-Prot 095399; Conlon JM et al. (1990) FEBS Lett, 266(1-2), 37-40). A region of 6 residues (CFWKYC) is cyclized by disulfide bridge formation and responsible for the biological activity of urotensin II (Coulouarn Y et al. (1998), PNAS U.S.A., 95(26), 15803-8; Itoh H et al. (1988), Eur. J. Pharmacol., 149, 61-6). This sequence is evolutionarily conserved from fish to human and shows high homology to somatostatin.

[0003] Human urotensin II precursor mRNA is actively expressed in many tissues of the central nervous system and the periphery, however expression in the spinal cord and medulla oblongata is by far higher than in any other tissue. urotensin II binds with high affinity to GPR14, a protein G-coupled orphan receptor with similarity to members of the somatostatin/opioid receptor family (Ames RS et al. (1999) Nature 401, 282-6; Nothacker HP et al. (1999), Nat. Cell Biol., 1, 383-385; Liu Q et al. (1999), Biochem. Biophys. Res. Commun., 266, 174-8; Mori M et al. (1999), Biochem. Biophys. Res. Commun., 265, 123-9). GPR14 is widely expressed in human tissues, including the left atrium and ventricle of the heart as well as in smooth muscle cells from the coronary artery and aorta. Whether human urotensin II is released locally or circulates in the plasma some distance from the target receptors remains to be elucidated.

[0004] Urotensin II exhibits various physiological and pharmacological effects, including general smooth muscle contracting activity [Davenport AP et al. (2000), TIPS 21, 80-82]. Both potency and efficacy of urotensin II induced vasoconstriction as measured *in vitro* is an order of magnitude higher than that of other peptides like endothelin-1, noradrenaline, serotonin and angiotensin II [Ames RS et al. (1999) Nature 401, 282-6; MacLean MR et al. (2000) Br. J. Pharmacol., 130, 201-204]. Systemic *in vivo*-administration of human urotensin II to anaesthetized monkeys caused marked systemic vasoconstriction, and resulted in severe myocardial contractile dysfunction and fatal circulatory collapse [Ames RS et al. (1999), Nature 401, 282-6]. In rat, the vasoconstrictive activity of human urotensin II lacks systemic pressure activity and is limited to the isolated thoracic aorta [Katano Y et al. (2000), Eur. J. Pharmacol., 402, 209-211; Bottrill FE et al. (2000) Br. J. Pharmacol., 130, 1865-1870]. Thus, the urotensin II peptide exhibits significant anatomical and functional differences in various species. urotensin II plasma values in humans as well as its potential role in human heart failure (CHF), however, have not yet been investigated.

[0005] One disadvantage of prior art methods for determining human urotensin II in body fluids is that antibodies raised against peptides from urotensin II tend to bind human proteins other than urotensin II.

[0006] Another disadvantage of prior art methods for determining urotensin II in plasma is that the measured levels of urotensin II do not relate to the known physiological effects of urotensin II and that the methods per se are not based on a biological model.

[0007] It was therefore the object of the invention to develop a both sensitive and specific immunoassay for the quantification of urotensin II in serum and plasma which can be applied to monitor the biological activity of this peptide.

[0008] This object has been achieved by a method as claimed in claim 1. Further embodiments of the invention are described in dependent claims 2 to 10.

[0009] In an embodiment of the invention the method of determining the biological active urotensin II in a sample of a body fluid, comprises contacting the sample with polyclonal and/or monoclonal antibodies which bind to a urotensin II structure having the sequence

CFWKYCX

SEQ ID NO: 1

wherein C is cysteine, F phenylalanine, W tryptophane, K lysine, Y tyrosine, wherein X is optional and either β -alanine, valine, or isoleucine and the region of CFWKYC is cyclized by a disulfide bridge formation.

[0010] In a preferred embodiment, the antibodies are elicited by immunisation with a peptide conjugate comprising the amino acid structure CFWKYC wherein the cysteines are cyclized by a disulfide bridge formation. More preferred are antibodies which have been elicited by immunisation with a cyclic peptide having the sequence

ETPDCFWKYC- β Ala-C,

SEQ ID NO: 2

with a disulfide bridge formed between Cys⁵ and Cys¹⁰, and comprising the artificial sequence β Ala-Cys, optionally conjugated with a carrier peptide.

[0011] In another method of the invention, the cyclic urotensin II peptide is bound to a stationary phase and the antibodies against urotensin II- β -Ala-Cys are purified by their specific binding to cyclic human urotensin II.

[0012] The method for determining purotensin II is preferably a competitive binding assay such as ELISA, RIA or EIA.

[0013] Method as claimed is being used in another embodiment of the invention for the diagnosis of cardiovascular diseases, hepatic syndrome, liver cirrhosis, diseases involving haemodynamic alterations associated with portal hypertension, pathological conditions involving abnormal levels of vaso-constrictive substances or for diagnosis of neurodegenerative diseases or traumatism of the spinal cord.

[0014] A further embodiment of the invention relates to a method of preparing cyclic urotensin II peptides comprising the step of cyclic oxidation of cysteins 5 and 10 following the synthesis of the primary urotensin II structure.

[0015] A detailed description of exemplary embodiments of the invention is provided below with reference to the following drawings, in which:

Fig. 1A is a chromatogram of the synthetic cyclic urotensin II peptide by analytical HPLC: Stationary phase: Nucleosil 300 5 μ C₁₈, eluent: A: 0.35% TFA/H₂O; B: 0.29% TFA/60% CH₃CN; gradient: 10-90% B in 36 min, λ =214 nm, 1 ml/min;

Fig. 1B is a matrix-assisted-laser-desorption-mass-spectrum of the synthetic cyclic urotensin II of the invention: N₀ of shots: 76, matrix: α -cyno-4-hydroxycinnamic acid;

Fig. 1C is a chromatogram of the synthetic (cyclic urotensin II)- β Ala-Cys peptide by analytical HPLC: Stationary phase: Nucleosil 300 5 μ C₁₈, eluent: A: 0.35% TFA/H₂O; B: 0.29% TFA/60% CH₃CN; gradient: 10-90% B in 36 min, λ =214 nm, 1 ml/min;

Fig. 1D is a matrix-assisted-laser-desorption-mass-spectrum of the synthetic (cyclic urotensin II)- β Ala-Cys which was used for immunisation: N₀ of shots: 87, matrix: α -cyno-4-hydroxycinnamic acid;

Fig. 2 is a diagram of calibration curves with different incubation schemes.

- ♦ 3 hours simultaneously standard and antibody
- sequentially 1 hour standard, 2 hours antibody
- x-- sequentially 1 hour standards, 1 hour antibody

Fig. 3 is a diagram showing the recovery of synthetic cyclic urotensin II peptide added to 10 different plasma samples;

Fig. 4 is a diagram showing the linearity of the U-II ELISA of the invention (Thirteen samples were assayed in a dilution of 1:10 and 1:30 in assay buffer);

Fig. 5 comparative analysis of 14 plasma samples; human urotensin II has been determined by the method of the invention and a commercial human urotensin II RIA assay (Phoenix Pharmaceuticals Inc.) with antibodies against linear human urotensin II. Weak correlation is seen.

EXAMPLE

Example 1 - Synthesis of urotensin II

[0016] Cyclic urotensin II peptide with the sequence

ETPDCFWKYC

(SEQ ID NO: 1)

(with a disulfide bridge formed between Cys⁵ and Cys¹⁰)

was synthesized in a stepwise manner using Fmoc-technology with an automated ECOSYN P peptide synthesizer (Eppendorf-Biotronik, Hamburg, Germany).

[0017] Starting with 1g (0.2 mMol) Fmoc-Cys (Trt) Trt-S-PHB-Tentagel-resin (Rapp Polymere, Tübingen, Germany), each Fmoc-amino acid derivative (0.8 mMol) was coupled to the resin using 0.26g (0.8 mMol) TBTU (2-(1H-benzotri-

azole-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate) and 1.1 ml DIEA-solution (20% diisopropylethylamine in dimethylformamide). The activation time was 4 min, the coupling time 30 min. The following protecting groups were used: cysteine: Trityl; lysine and tryptophane: t-butyloxycarbonyl; tyrosine and threonine: t-butyl; aspartic- and glutamic acid: O-tert-butylester. After introduction of the last amino acid, the Fmoc-group was removed using 25% piperidine in dimethylformamide (20 min) and the Boc-group was introduced using Boc₂O/DIEA in dichloromethane (10 fold excess; 1h, room temperature). The fully protected peptide was removed from the resin using a mixture of acetic acid, trifluoroethanol, dichloromethane (40/10/50 ml) by stirring for 1h at room temperature. After removal of the solvents, the peptide was precipitated from dichloromethane with petrolether yielding 340 mg. Oxidation of the peptide (0.16 mMol) was achieved using 0.3 g iodine dissolved in hexafluor-2-propanol/dichloromethane (5/35 ml). The substrate in 10 ml dichloromethane and 5 ml hexafluor-2-propanol was added dropwise to the iodine solution. After 9 min, the reaction was stopped by addition of an ice-cold solution of ascorbic acid (0.92g) and 0.7g ammonium acetate. After separation of the organic layer, the water phase was extracted twice with chloroform. The combined organic phases were dried with sodium sulfate. The final deprotection of the remaining protecting groups was performed using a mixture of trifluoroacetic acid/thioanisole/anisole/trisopropylsilane (12/0.6/0.3/0.1 ml) for 2 h at room temperature. After filtration from the resin, the peptide was precipitated with diethylether. The precipitated crude peptide was redissolved in 20% acetic acid and subsequently lyophilized. The yield was 240 mg. Finally, the peptide was purified by semipreparative HPLC using a Nucleosil 300 5 μ C₁₈-column (Macherey-Nagel, Düren, Germany). The gradient was from 10-90% B in 36 min (A: 0.35% TFA/H₂O; B: 80% CH₃CN, 0.29% TFA/H₂O). Flowrate was 3.5 ml/min, detection at λ 214 nm.

[0018] For coupling of the urotensin II to bTG, the peptide with the following sequence was used

ETPDCFWKYC- β Ala-C

SEQ ID NO: 2

(with a disulfide bridge formed between Cys⁵ and Cys¹⁰). The synthesis of this peptide was performed in a similar manner (0.2 mMol scale) as for the former one except that for the protection of Cys⁵ and Cys¹⁰, the Mmt (Methoxytrityl)-group was used. Following synthesis, the peptide and the Mmt-groups were removed simultaneously from the resin. The disulfide bridge was formed by oxidation with a mixture of dimethylsulfoxide/dimethylformamide/water (30/15/5; 100 ml) over night at room temperature. After removal of the solvents, the peptide was precipitated from the dichloromethane by adding petrolether. Removal of the remaining protection groups and HPLC purification was done as described above.

[0019] Both peptides were fully analyzed and characterized by analytical HPLC and mass-spectrometry (see Figs. 1A-D).

Example 2 - Immunisation

[0020] The cyclic urotensin II peptide coupled to betathromboglobulin (bTG) was emulsified in ABM® (Lenaris GmbH, Wertheim, Germany) and used to immunize four white Zealand rabbits. Test bleeds were taken one week after boosts and rabbits were boosted three times in an eight week period.

[0021] The immunological response was monitored by an ELISA with urotensin II peptides immobilized on microtiter plates. Addition of serial dilutions of the rabbit antisera was followed by horseradish peroxidase (HRPO) labelled donkey anti rabbit Ig (DAKO, Germany). To visualize the immunoreactivity, the ready to use single component TMB substrate was employed (BioFX, USA). The bleed obtained after the third boost was used for further development of the assay.

Example 3 - Antibody Purification

[0022] urotensin II specific antibodies were purified from antiserum using immunoaffinity chromatography on HiTrap minicolumns (Pharmacia, Sweden). According to the manufacturer's protocol, 0.3 g of synthetic cyclic urotensin II peptide was bound to the resin. 5 ml of antiserum were filtered through a 0.45 μ m Millex-Filter (Millipore, USA) diluted with 2 volumes of 50 mM borate buffer pH 7.0 and loaded at room temperature onto the HiTrap minicolumn at a flow rate of 0.5 ml/min. After washing the resin, specific antibodies were eluted with 0.1 M citrate buffer pH 1.7 at a flow rate of 1 ml/min. Elution was monitored by UV detection at 280 nm, and fractions of 0.5 ml were collected onto 0.5 ml of 0.5 M borate buffer pH 10 to ensure immediate neutralisation. Concentration of the specific Ig was determined with a modified μ BCA protein assay (Pierce, The Netherlands).

Example 4 - Assay Development

[0023] Microtiter plates (Nunc Maxisorp High Binding, NUNC, Denmark) were coated for 3 h at room temperature with 0.1 ml of 0.05M borate buffer, pH 9.6, containing 250 pg of synthetic cyclic urotensin II peptide. Remaining binding

fraction, $17\pm 3\%$) who demonstrated DCM ($n=3$) or IHD ($n=3$) and underwent partial ventriculectomy or transplantation.

Right atria and vessels

[0041] Samples from right atrium ($n=8$), mammary artery ($n=6$), and saphenous vein ($n=6$) were obtained from patients with normal heart function; vascular specimens ($n=4$ each) were also taken from patients with CHF ($n=4$; LV ejection fraction, $20\pm 3\%$). All these patients underwent bypass surgery. We furthermore used samples from dilated right atria of DCM patients ($n=4$; LV ejection fraction, $18\pm 2\%$) who underwent partial ventriculectomy. In all patients, heart function had been assessed by preoperative catheterization.

Analysis of mRNA

[0042] We isolated total RNA and performed reverse transcription according to standard procedures. PCR amplification of single-stranded cDNA was then performed utilizing primer for prepro urotensin II as described in Coulouarn Y et al. (1998), Proc. Natl. Acad. Sci. USA., 95, 15803-8 (TIB MOLBIOL), and for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

SEQ ID NO: 3

upstream: 5' TGA AGG TCG GAG TCA ACG GAT TTG GT 3'

SEQ ID NO: 4

downstream: 5' CAT GTG GGC CAT GAG GTC CAC CAC 3'

(Clontech).

Southern blot hybridization was conducted for quantization of the amplified sequences using radioactively labeled specific oligo prepro U-II:

SEQ ID NO: 5

5' CCA TAC AAG AAA CGT GAG ACT CCT GAT TGC 3'

Subsequent to autoradiography, data were normalized to GAPDH mRNA expression.

Statistics

[0043] Data are presented as mean \pm SEM unless otherwise indicated. An error probability of $P<0.05$ was regarded as significant. Baseline values for unpaired data (haemodynamic parameters, mRNA data) were compared using the Kruskal-Wallis ANOVA or the Mann-Whitney rank sum test. Baseline values paired data (peptide concentrations at different sites of measurement) were compared using the Friedmann ANOVA on ranks. Differences between groups over time (haemodynamics and peptide levels) were analyzed with a nonparametric ANOVA for repeated measures. In each case, a multiple-comparison procedure with Bonferroni-Holm adjustment of P was carried out after global testing. (Itoh H et al. (1988), Eur. J. Pharmacol., 149, 61-6.)

Results

Haemodynamics

[0044] Table 2 illustrates the significant deterioration of baseline haemodynamics with the progression of CHF. In severe CHF, SNP treatment (Table 2) induced significant haemodynamic improvement: i.e., decreases in pulmonary arterial pressure ($-41\pm 4\%$), pulmonary capillary wedge pressure ($-55\pm 3\%$), systemic ($-63\pm 5\%$) and pulmonary vascular resistance ($-60\pm 3\%$), as well as an increase in cardiac index ($-78\pm 3\%$). Maximum effects were already achieved within the first 2 h of treatment. In moderate CHF, only the decreases in mean arterial pressure ($-32\pm 2\%$) and systemic vascular resistance ($-53\pm 3\%$) were significant.

TABLE 2

Haemodynamics at Baseline and During Vasodilator Therapy							
Time(h)	HR	MAP	MPAP	PCWP	CI	SVR	PVR
Severe CHF							
0	91±4	83±2	46±2 ^b	33±2 ^b	1.8±0.2 ^b	1921±137	356±39 ^b
24	86±3	61±3 ^d	27±2 ^d	15±1 ^d	3.2±0.1 ^d	720±21 ^d	144±15 ^d
Moderate CHF							
0	84±4	87±5	22±2	14±2	2.9±0.3	1602±35 ^c	160±13 ^c
24	77±8	60±2 ^d	19±2	11±2	3.6±0.7	757±72 ^d	106±46
Controls							
0	80±3	92±2 ^a	14±1 ^a	10±1 ^a	3.5±0.1 ^a	707±26 ^a	92±4 ^a

HR, Heart rate; MAP, mean arterial pressure (mm Hg); MPAP, mean pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; CI, cardiac index (L/min/m²); SVR/PVR, systemic /pulmonary vascular resistance (dyn/s/cm⁵). P<.05; ^a, severe CHF vs. controls; ^b, severe vs. moderate CHF; ^c, moderate CHF vs. controls; ^d, vs. baseline.

Plasma Urotensin II

[0045] Depending on the sites of measurement, baseline levels in controls ranged between 8.4±2.4 and 1.11±4.0 ng/mL (V). In moderate CHF, values were measured between 8.5±3.0 (LV) and 10.5±2.2 ng/mL (V); and in patients with severe CHF, we determined levels between 11.0±4.0 (CS) and 13.0±3.2 ng/mL (PA). We selected significant differences neither between the 3 groups nor between sites of measurement within a single group of patients.

[0046] During vasodilator therapy, the significant haemodynamic improvement achieved in the patients with severe CHF did not result in changes of circulating urotensin II over 24 h (Fig. 2b). Patients with moderate CHF similarly exhibits no alteration of plasma urotensin II during treatment (data not shown).

Urotensin II mRNA in myocardium and vasculature

[0047] The expression of prepro urotensin II mRNA in tissue samples obtained from patients with preserved heart function and patients with end-stage CHF. We detected different gene expression neither in right atrial (control, 1.0±0.2 vs. CHF, 1.3±0.2 arbitrary units) nor in left ventricular myocardium (1.0±0.3 vs. 0.7±0.3 units). Similarly, prepro urotensin II mRNA levels in mammary arteries and saphenous veins from controls were comparable to those detected in end-stage CHF (mammary artery, 1.0±0.4 vs. 1.3±0.4 units; saphenous vein, 1.0±0.3 vs. 0.8±0.2 units).

Discussion

[0048] Owing to its potent vasoconstrictor effects, urotensin II represents a candidate regulator of vascular tone in physiological and pathophysiological states. Plasma urotensin II seems independent of the severity of CHF and shows no response to haemodynamic improvement in severe CHF. Significant differences between sites of measurement - indicating net release or consumption across pulmonary circulation (LV minus PA levels), coronary (CS-LV), or peripheral vascular beds (V-A) - were found in none of the groups. The gene expression of urotensin II in myocardial and vascular samples from end-stage CHF was comparable to that determined in control specimens.

[0049] Until now, no studies have been carried out to investigate the role of urotensin II in CHF. Judging from our findings, involvement of urotensin II in the pathophysiology of human CHF cannot be ruled out. First, because no relevant spare receptor reserve seems to exist in the urotensin II system (Itoh H et al. (1988), Eur. J. Pharmacol., 149, 61-6.) and because urotensin II behaves as a "pseudo-irreversible" ligand like endothelin-1 (Douglas SA et al. (2000), Br. J. Pharmacol., 131, 1262-1274). Even modest changes in the expression of urotensin II receptors may have profound effects on contractile efficacy and, accordingly, on pathophysiological impact. The new test system for cyclic urotensin II according to the invention will serve to clarify these items. Second, urotensin II exhibits a unique "anatomically restricted" contractile profile: In rats and dogs, it is a selective aortocoronary constrictor (Douglas SA et al. (2000), Br. J. Pharmacol., 131, 1262-1274) although it has a broader spectrum of target vessels in primates and humans (Maguire JJ et al. (2000), Br. J. Pharmacol., 131, 441-6). However, human mammary arteries and saphenous veins

are highly sensitive to urotensin II which proves the existence of a functional urotensin II system in the vessels utilized in our study. It appears therefore unlikely that a site-restricted vascular regulation in CHF remains in the type of vessels examined.

5 Example 6 - *Determination of plasma urotensin II levels in patients with cirrhosis*

Patients and Methods

10 **[0050]** Patients with portal hypertension are characterized by splanchnic vasodilation and activation of endogenous vasoconstrictor. Since urotensin II is the strongest endogenous vasoconstrictor of large arterial vessels discovered so far [Ames RS et al. (1999), *Nature* **401**, 282-6] and in contrast relaxes rat mesenteric resistance vessels (Botrill FE et al. (2000), *Br. J. Pharmacol.*, **130**, 1865-70) we determined plasma levels in patients with cirrhosis and healthy control persons.

15 **[0051]** Plasma urotensin II levels were measured by ELISA in accordance with the invention in 20 patients with cirrhosis and portal hypertension (wedged hepatic venous pressure gradient = 19 ± 1 mmHg) and in 10 aged matched healthy controls. Additionally, plasma urotensin II levels were compared between hepatic venous and portal venous blood of 10 patients with cirrhosis prior to TIPS insertion.

Results

20 **[0052]** urotensin II plasma levels were significantly higher in cirrhotic patients (10640 ± 1751 pg/ml) compared to control persons (3921 ± 889 pg/ml, $p < 0.005$). In cirrhotic patients undergoing TIPS implantation hepatic venous urotensin II plasma levels were significantly higher (14756 ± 3500 pg/ml) compared to portal venous plasma levels (11931 ± 2973 pg/ml, $p < 0.02$).

25 **[0053]** In conclusion, urotensin II plasma levels are increased in patients with cirrhosis. The gradient between portal and hepatic venous urotensin II levels probably indicates the hepatic origin of urotensin II in cirrhosis. Thus, the plasma level of urotensin II plays a role in hemodynamic alterations associated with portal hypertension.

30 **[0054]** Plasma urotensin II levels were also measured in patients having a liver stent. Blood samples were taken from V. portae (entry) and V. cava inferior (exit) of the liver and measured and it was found that the levels of urotensin II in blood was increased after passage of the liver up to 30fold.

[0055] Interestingly, it was also found that plasma levels of urotensin II in healthy persons are significantly increased by smoking.

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SEQUENZPROTOKOLL

<110> IMMUNDIAGNOSTIK AG

ABC Armbruster Biochemicals

<120> Method of Determining Urotensin II in Body Fluids and
Diagnosis of Cardiovascular Diseases

<130> HL-1195.001/UMB

<140>

<141>

<150> 01105418.6

<151> 2001-03-12

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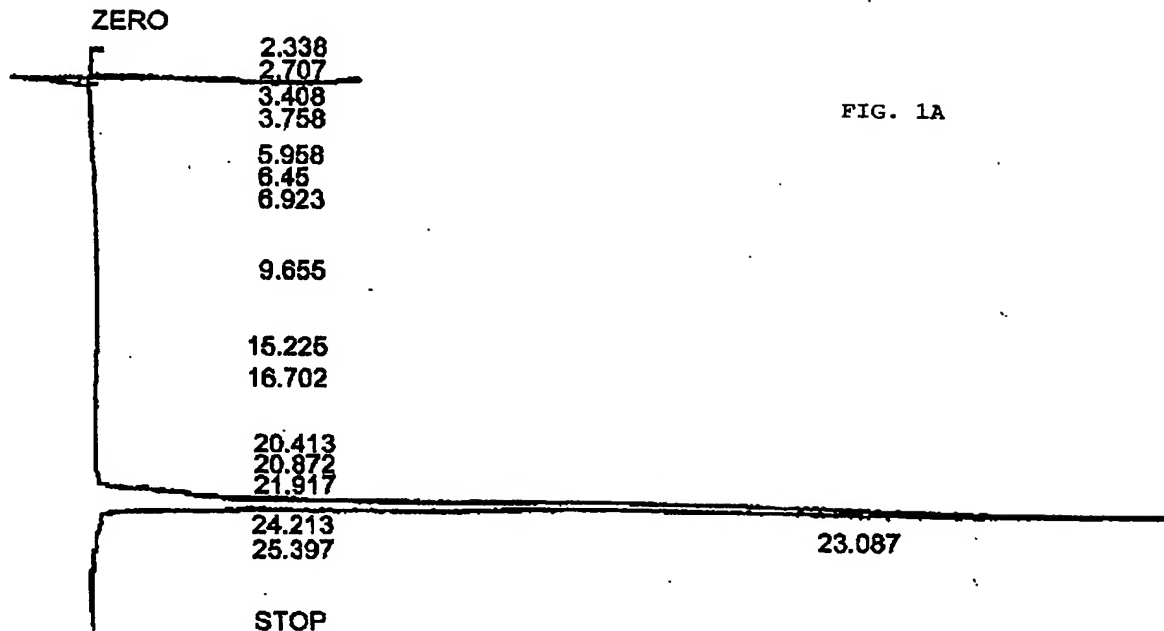
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6. Method as claimed in any preceding claim 1 to 5, wherein the antibodies against urotensin II have been purified by their specific binding to the cyclic structure of urotensin II.
7. Method as claimed in any preceding claim 1 to 5, which is an ELISA, RIA or EIA.
8. Method as claimed in any preceding claim 1 to 7 for a diagnosis of cardiovascular diseases, hepatorenal syndrome, cirrhosis, pathological conditions involving haemodynamic alterations, pathological conditions involving vaso-constrictive substances.
9. Method as claimed in any preceding claim 1 to 7 for a diagnosis of neurodegenerative diseases or traumatism of the spinal cord.
10. Method of preparing cyclic urotensin II peptides comprising the step of cyclic oxidation of cysteins 5 and 10 following the synthesis of the primary urotensin II structure.

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00/03/28

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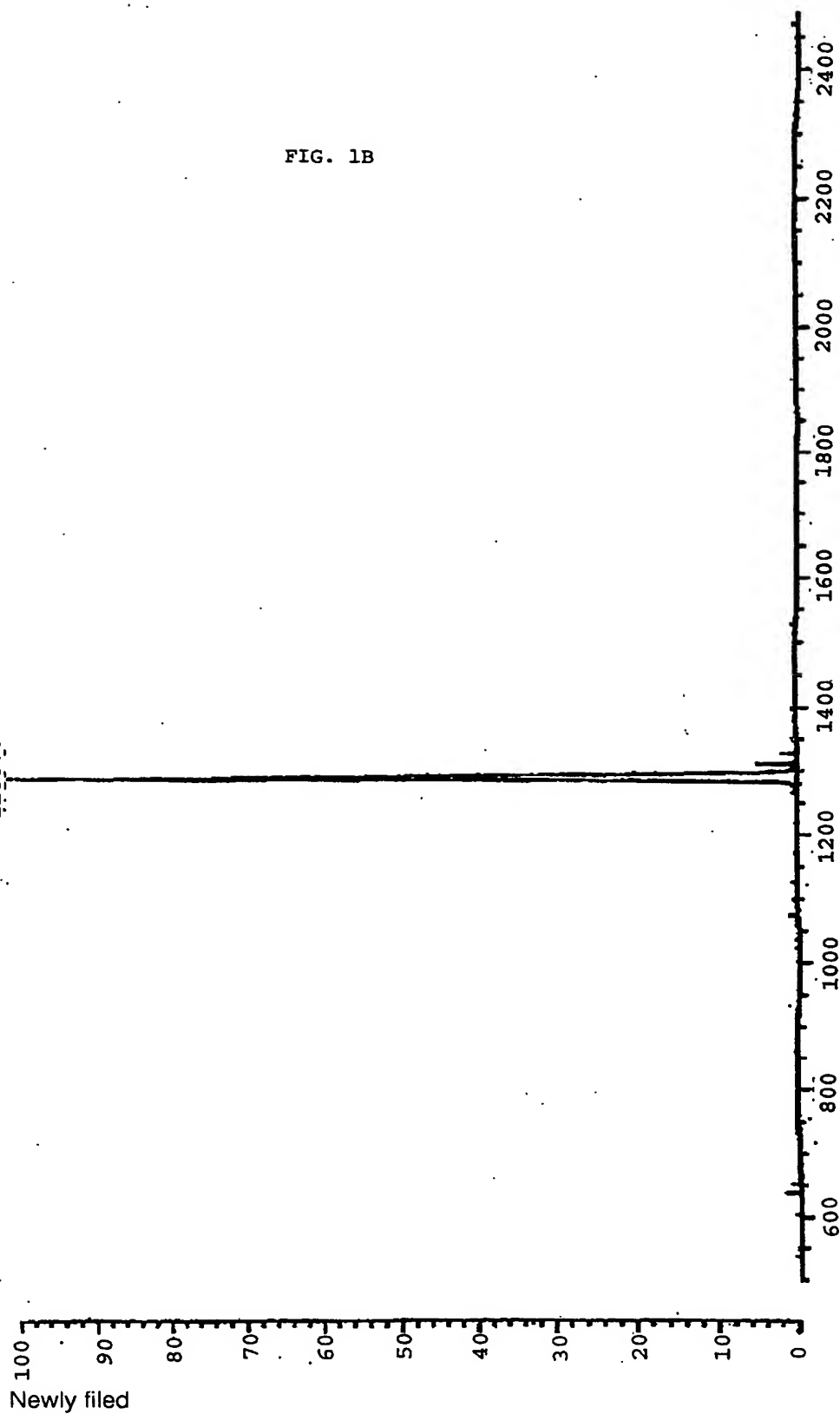
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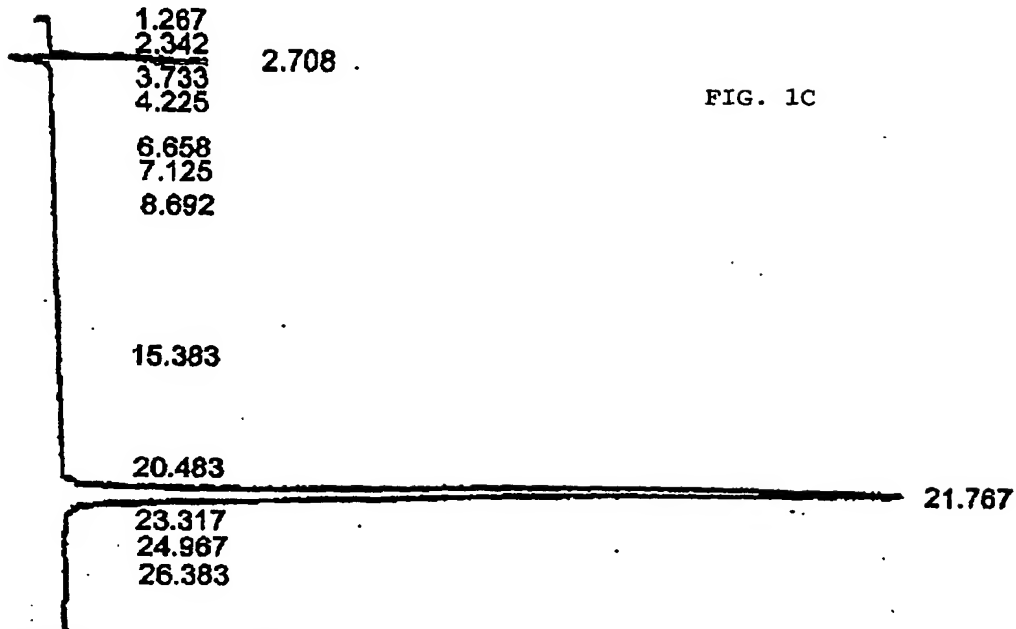
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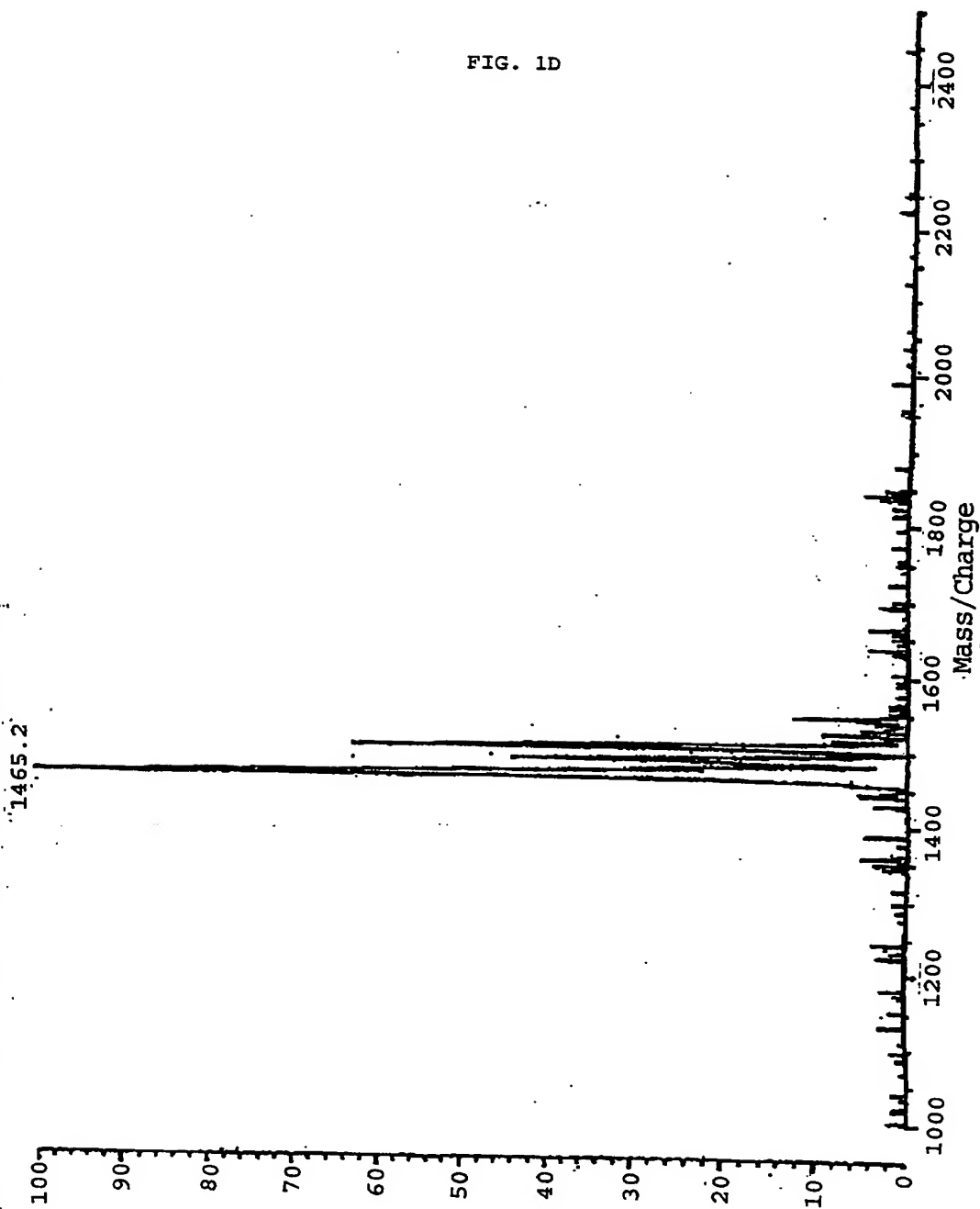


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6	6.658	661240	V	9.7898	
7	7.125	226595	V	3.3548	
8	8.692	1569988	V	23.2439	
9	15.383	649381	V	9.6142	
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Urotensin b-Ala-Cys
Data: <Untitled>.16 24 Mar 2000 10:43 Cal: adaamy 15 Dec 1999 13:37
Kratos Kompact MALDI 2 V5.2.4: + Linear High Power: 87
%Int. 100%=9 mV [sum= 488 mV] Profiles 1-50 Smooth Av 1



(19)



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(11)

EP 1 241 479 A3

(12)

EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
14.01.2004 Bulletin 2004/03

(51) Int Cl.⁷: **G01N 33/74**, G01N 33/543,
C07K 14/575, G01N 33/53

(43) Date of publication A2:
18.09.2002 Bulletin 2002/38

(21) Application number: **01106463.1**

(22) Date of filing: **23.03.2001**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

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(30) Priority: **12.03.2001 EP 01105418**

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(54) **Method of determining urotensin II in body fluids and diagnosis of cardiovascular diseases**

(57) Method of determining the biological active urotensin II in a sample of a body fluid, comprising contacting the sample with polyclonal and/or monoclonal antibodies which have been elicited by immunisation with a cyclic peptide having the sequence:

ETPDCFWKYC-βAla-C,

SEQ ID NO: 2

having a disulfide bridge formed between Cys⁵ and Cys¹⁰, and comprising the artificial sequence βAla-Cys, optionally conjugated with a carrier peptide. The obtained antibodies are then either purified or characterised by their binding to cyclic human urotensin II and can be used in a binding assay for a diagnosis of cardiovascular diseases, heptareneal syndrome, cirrhosis, diseases involving haemodynamic alterations, pathological conditions involving vasoconstrictive substances.

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European Patent
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EUROPEAN SEARCH REPORT

Application Number
EP 01 10 6463

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The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 14 November 2003	Examiner Trommsdorff, M
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03 82 (P04001)

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

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The members are as contained in the European Patent Office EDP file on
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14-11-2003

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